

AD_____

Award Number: W81XWH-06-1-0496

TITLE: Killing Breast Cancer Cells through Activation of the Apoptosome

PRINCIPAL INVESTIGATOR: Sally Kornbluth, Ph.D.

CONTRACTING ORGANIZATION: Duke University
Durham, NC 27708

REPORT DATE: June 2008

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.					
1. REPORT DATE (DD-MM-YYYY) 01-06-2008		2. REPORT TYPE Annual		3. DATES COVERED (From - To) 8 May 2007 – 7 May 2008	
4. TITLE AND SUBTITLE Killing Breast Cancer Cells through Activation of the Apoptosome				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-06-1-0496	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Sally Kornbluth, Ph.D. E-Mail: kornb001@mc.duke.edu				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Duke University Durham, NC 27708				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Cells die in response to chemotherapeutic agents by activating a mitochondrial cell death pathway. This pathway leads to activation of proteases known as caspases through the release of cytochrome c from mitochondria. Once released, cytochrome c engages a cytoplasmic receptor known as Apaf-1, which oligomerizes and activates caspase 9. We demonstrated that breast cancer cells have apoptosomes which are hypersensitive to cytochrome c. Thus we have proposed to develop cytoplasmic variants of cytochrome c and/or small molecule cytochrome c mimetics to activate the apoptosome in breast cancer cells.					
15. SUBJECT TERMS No subject terms provided.					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	7	19b. TELEPHONE NUMBER (include area code)

Table of Contents

	<u>Page</u>
Introduction.....	4
Body.....	4
Key Research Accomplishments.....	7
Reportable Outcomes.....	7
Conclusion.....	7
References.....	
Appendices.....	

Body of report:

Introduction

In response to chemotherapeutics, breast cancer cells initiate a cell death program culminating in the release of cytochrome c from mitochondria, binding of cytochrome c to a protein known as Apaf-1, and consequent Apaf-1-induced activation of a cell death protease known as caspase 9. We had reported previously that breast cancer cells were more sensitive to cytochrome c-induced apoptosis than their normal counterparts. Based on these findings, we wished to develop cytochrome c mimetic therapeutics for the treatment of chemoresistant breast tumors. We proposed both to modify cytochrome c itself as a potential therapeutic and screen for small molecules that might mimic cytochrome c.

Our original statement of work contained the following aims:

- Task 1:** Construction of cytochrome c and heme lyase variants allowing the production of cytoplasmically active cytochrome c (months 1-6)
- Task 2:** Testing of cytoplasmic cytochrome c/heme lyase in tissue culture and mice (months 12-20)
- Task 3:** Identification and testing of small molecule post-mitochondrial caspase activators (months 21-36)

As detailed in last year's report, we began with **Task 3** and isolated several potentially interesting small molecules that promoted cell death. Unfortunately, these compounds turned out to kill cells in an Apaf-1-independent manner. While these may be of interest to pursue, they are not likely to be breast cancer selective in their killing. We have now prepared additional cytosolic lysates and have introduced a secondary screen using Apaf-1 null lysates (in which compounds of interest should not induce caspase activation).

In an alternative approach, we have also developed a novel single cell screen for regulators of caspase activity using the near-infrared caspase activity indicator (the caspase-3 IRDye, available from LI-COR Biosciences Inc) and the *Xenopus* oocyte system. Injection of these large cells is technically simple and thousands of cells can be injected in 4-5 hours. As shown in Fig. 1, oocytes injected with this indicator and cytochrome c very rapidly cleave the substrate to produce an infrared signal detected by the LI-COR Odyssey reader. This signal is quite robust and enduring; caspase activation could be detected as little as 3 minutes after injection and signal persisted for 24 hours or more (Fig. 2). Similar results were obtained when we injected the pro-apoptotic Bcl-2 family member, t-Bid. Moreover, the signal was inhibited by co-injection of oocytes

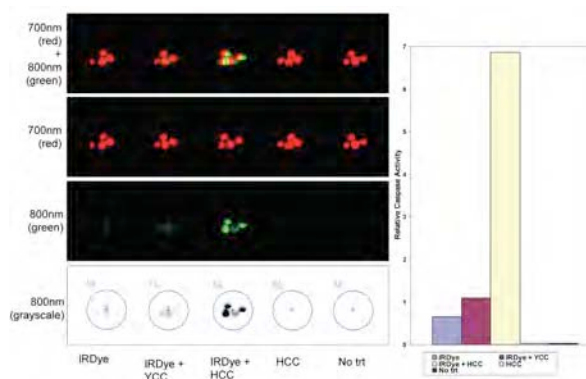


Figure 1. Fluorescence in oocytes co-injected with the IRDye® and cytochrome c. Oocytes were injected with either IRDye, IRDye and yeast cytochrome c (YCC), IRDye and horse cytochrome c (HCC), HCC alone, or nothing (No trt). Oocytes were imaged using the LI-COR Odyssey® thirty minutes after injection. Resulting images are shown on the left, demonstrating oocyte autofluorescence in the red channel (700nm) and IRDye signal in the green channel (800nm). 800nm signal from each group of oocytes was quantitated in the grayscale image (blue circles), and a graph from the obtained values is displayed on the right. As shown, significant signal is obtained only oocytes co-injected with IRDye and HCC.

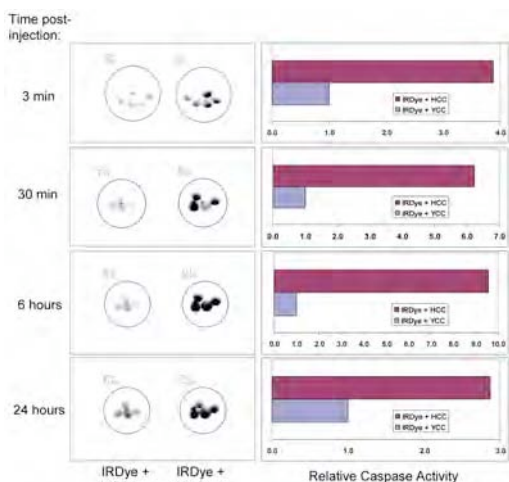


Figure 2. Oocyte fluorescence can be detected from three minutes to twenty-four hours after injection. Oocytes were microinjected with either IRDye + YCC or IRDye + HCC. Images of the injected oocytes were obtained on the LI-COR Odyssey® over time. Quantitated grayscale images of the 800nm signal are shown on the left for several time points, with corresponding graphs displayed on the right. Significant 800nm signal is obtained from as early as three minutes post-injection and is maintained for at least twenty-four hours selectively in the HCC-injected oocytes.

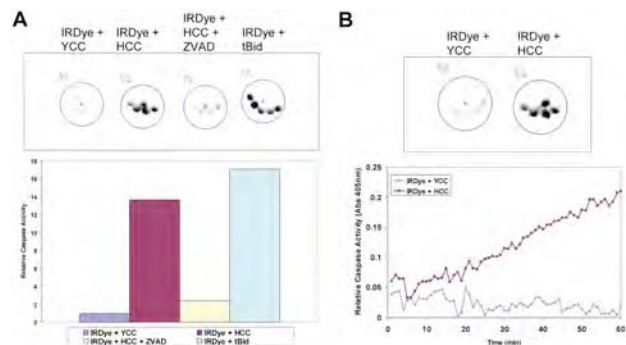


Figure 3. Fluorescence in IRDye-injected oocytes is due to activation of effector caspases. **A**, Oocytes were injected with either IRDye + YCC, IRDye + HCC, IRDye + HCC + the general caspase inhibitor ZVAD-FMK (ZVAD), or IRDye + a truncated form of Bid (tBid). A quantitated grayscale image of the 800nm signal and the corresponding graph are displayed. **B**, Oocytes injected as indicated (top) were flash-frozen thirty minutes following injection, lysates were prepared and caspase activation was measured using a colorimetric substrate (bottom).

with the caspase inhibitor zVAD-fmk (Fig 3). We have acquired a library of bioactive compounds and intend to test groups of these (in pools) by manual injection to determine if we can identify pools that either rapidly induce apoptosis or that inhibit cytochrome c-induced apoptosis. Pools would then be deconvoluted by breaking down into smaller pools, etc. This is far more labor intensive than the automated screens we have ongoing, but this may be a more sensitive assay, allowing us to detect novel small molecule apoptotic modulators more readily.

During this past year, we have been focused on **Task 1** to produce cytochrome c and heme lyase variants that are cytoplasmic. Initially, we sought to do this systematically through the identification of residues important for mitochondrial import of these proteins. However, careful reading of the literature suggested that mutations disrupting mitochondrial import might also disrupt protein function. Therefore, we took a distinct approach, making fusion proteins that would alter either the N- or C- termini of the proteins, hoping that these might impede import, while allowing retention of function. Towards this end, we utilized an EGFP-cytochrome c, a cytochrome c-EGFP, an mCherry-heme lyase. These fusions offered the added advantage that we could visualize these proteins through fluorescence microscopy and/or sort cells expressing these proteins by fluorescence activated cell sorting. Interestingly, it had been reported previously that GFP-cytochrome c and cytochrome c-GFP could localize to mitochondria. However, we found that this was true in only a minor subset of cells expressing this construct. Rather, the majority was cytoplasmic (Fig. 4). Similar results were obtained with mCherry-heme lyase (see red cells in Fig. 5). We have begun experiments described in **Task 2**, expressing the cytoplasmic variants of cytochrome c and heme lyase in a variety of cell types to determine if this induces apoptosis. Preliminary results are promising; we see green

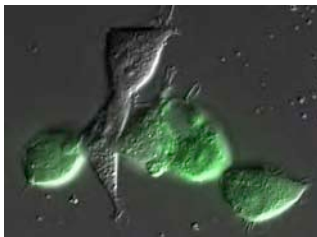
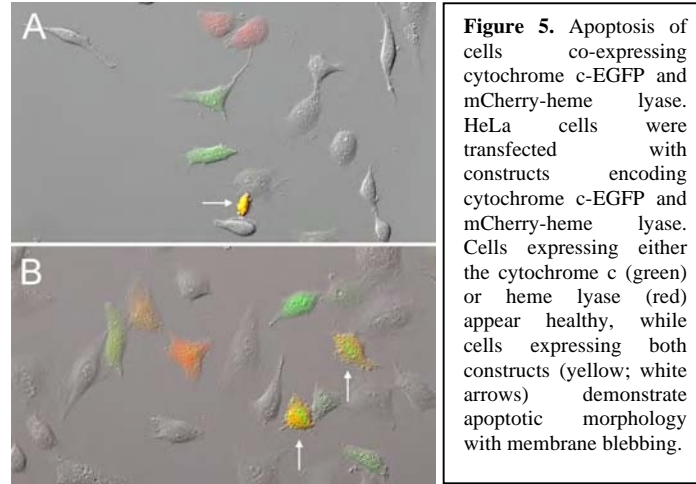


Figure 4. Cytosolic expression of cytochrome c fused to EGFP. HeLa cells were transfected with a construct encoding cytochrome c-EGFP and then visualized twenty-four hours later using fluorescence microscopy. Green fluorescence is detected throughout the cytosol in transfected cells. A similar staining pattern is observed in cells expressing EGFP-cytochrome c.

cells and red cells singly infected with these constructs that appear healthy. However, yellow cells (shown in Fig 5) appear to exhibit apoptotic morphology. These experiments will be repeated and we will also confirm, via caspase assays, that the morphology is truly indicative of apoptotic cell death. If these results appear promising, we will proceed to the originally described animal experiments.



Currently **no reportable outcomes**, but we have developed the necessary tools to proceed.

Key research accomplishments:

- Generation of cytoplasmic cytochrome c
- Generation of cytoplasmic heme lyase
- Initial assays on co-expression of cytoplasmic cytochrome c and heme lyase
- Development of a new assay for small molecule screening

Conclusions: We have developed the tools detailed in Task 1 and will proceed to the phenotypic testing of these constructs as detailed in Task 2, while continuing our original Task 3 experiments.